PROTOCOL FOR THE PROPOSED NEW CONFIRMATORY TEST FOR SPRING VIRAEMIA OF CARP

1.2.2 Confirmatory identification methods

Polymerase chain reaction

The genome of spring viraemia of carp virus (SVCV) consists of a single strand of RNA of approximately 11 kb, with negative polarity. Amplification of a 714 bp fragment of SVCV cDNA is performed using primers derived from sequences of the region coding for the glycoprotein gene: 5'-TCT-TGG-AGC-CAA-ATA-GCT-CAR*R*-TC-3' (SVCVF1) and 5'-AGA-TGG-TAT-GGA-CCC-CAA-TAC-ATH-*ACN*-CAY*-3' SVC R2), according to the method of Stone *et al.* (2003).

Total RNA is extracted from 100 µl of viral supernatant from infected EPC-cells using the Trizol Reagent™ and dissolved in 40 µl molecular biology grade water according to the method of Strømmen & Stone (1997).

For cDNA synthesis, a reverse transcription reaction is performed at 37°C for 1 hour in a 20 µl volume consisting of 1 x M-MLV RT reaction buffer (50 mM Tris, pH 8.3, 75 mM KCL, 10 mM DTT, 3 mM $MgCl_2$) containing 1 mM dNTP, 100 pmol SVCV R2 primer, 20 units M-MLV reverse transcriptase (Promega, Southampton UK) and 1/10 of the total RNA extracted above. Polymerase chain reaction (PCR) is performed in a 50 µl reaction volume 1 x PCR buffer (50 mM KCl, 10 mM Tris/HCl, pH 9.0, and 0.1% Triton X-100) containing 2.5 mM $MgCl_2$, 200 µM dNTPs, 50 pmol each of the SVCV R4 and SVCV F1 primers, 1.25 units of Taq DNA polymerase, and 2.5 µl reverse transcription reaction mix. The reaction mix was overlaid with mineral oil and subjected to 35 temperature cycles of: 1 minute at 95°C, 1 minute at 55°C and 1 minute at 72°C followed by a final extension step of 10 minutes at 72°C. Amplified DNA (714 bp) is analysed by agarose gel electrophoresis

If the cytopathic effects in culture are not extensive it is possible that a product will not be generated using a single round of amplification. To avoid such problems use the semi-nested assay using primers:

5'-TCT-TGG-AGC-CAA-ATA-GCT-CAR*R*TC-3' (SVCVF1) and

5'-CTG-GGG-TTT-CCN*CCT-CAA-AGY*TGY*-3' (SVC R4) according to Stone *et al.* (2003). The second round of PCR is performed in a 50 µl reaction volume 1 x PCR buffer (50 mM KCl, 10 mM Tris/HCl, pH 9.0, and 0.1% Triton X-100) containing 2.5 mM $MgCl_2$, 200 µM dNTPs, 50 pmol each of the SVCV R2 and SVCV F1 primers, 1.25 units of Taq DNA polymerase, and 2.5 µl of the first round product. The reaction mix is overlaid with mineral oil and subjected to 35 temperature cycles of: 1 minute at 95°C, 1 minute at 55°C and 1 minute at 72°C followed by a final extension step of 10 minutes at 72°C. Amplified DNA (606 bp) is analysed by agarose gel electrophoresis

All amplified products are confirmed as SVCV in origin by sequencing, and the SVCV subtype (Ia-Id) is identified using a BLAST search (http://www.ebi.ac.uk/blastall/index.html) .

*SVCV primer annealing sites were identified by the alignment of the published amino acid sequences for the glycoprotein of SVCV (Bjorklund *et al.* 1996; Genbank accession no. U18101), and the vesicular stomatitis virus (VSV) New Jersey (Gallione & Rose 1983; Genbank accession no. V01214), and Piry strains (Genbank accession no. D26175). Primers were then designed to anneal to the regions encoding the conserved amino acids using the published sequence for SVCV (Bjorklund *et al.* 1996) as a skeleton, and introducing degenerate bases at the 3' termini to allow for potential differences in codon usage. The appropriate IUB codes have been used where appropriate.

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